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Award Number: W81XWH-05-1-0060

TITLE: Methods to Protect from Skeletal Cardiovascular Insufficiency: Improving Soldier Performance in Adverse Environments

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REPORT DATE: November 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01/11/06		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 Nov 05 – 31 Oct 06	
4. TITLE AND SUBTITLE Methods to Protect from Skeletal Cardiovascular Insufficiency: Improving Soldier Performance in Adverse Environments				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0060	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Bruce T. Liang, MD; Michael Azrin MD; William White MD; George Mansoor MD; Mozafareddin Karimeddini MD; Kimberly Dodge PhD; Marybeth Barry APRN; Pat Tyrell RN; Jessica Kupiec BA; Rubio Wang, M.D. PhD; Jing-Gang Zheng, M.D.; Alexander Yang BA MS; Jayne Schumacher BS RN E-Mail: bliang@uchc.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Connecticut Health Center Farmington CT 06030-0002				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The main objective of the project is to study the cardiovascular and skeletal muscle protective actions of purines. The pre-clinical animal work has resulted in the establishment of a quantitative mouse hindlimb model of ischemia-reperfusion injury and elucidation of the role of adenosine receptor subtypes in mediating a skeletal muscle protective effect. The study demonstrated for the first time a novel protective action of the adenosine A3 receptor. It also confirmed, in this model, a protective effect of the adenosine A1 receptor. The significance is that the studies identified the adenosine A3 receptor as a new therapeutic target for the treatment of skeletal muscle ischemia-reperfusion injury. Agonists at the adenosine A3 receptor are potentially novel agents to ameliorating skeletal muscle injury. The clinical works have also begun to test the genetic polymorphism of adenosine transporter and its biological significance. At this point, we have identified several polymorphisms, some are non-synonymous and cause missense mutations.					
15. SUBJECT TERMS Skeletal muscle protection, heart protection, adenosine					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 67	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION

Development of methods to protect from skeletal and cardiovascular insufficiency is the main objective of the current research. Adenosine is a potent cyto-protective hormone released during ischemia. Adenosine can protect the heart and skeletal muscle from ischemia-reperfusion injury. However, the subtype of adenosine receptors mediating this protection in skeletal muscle is not well understood. A specific objective here is to determine the role and subtype of adenosine receptors that mediate skeletal muscle protection using a quantitative mouse hindlimb ischemia-reperfusion injury model. The goal of the clinical research is to test the presence of genetic polymorphisms in the adenosine transporter gene and determine whether it is associated with an altered dipyridamole responsiveness. The hypothesis is that some or all of these polymorphisms are associated with a decreased responsiveness to dipyridamole and that increasing the dose of dipyridamole will overcome the relative non-response.

BODY:

The pre-clinical animal research during this period can be summarized as follows.

Adenosine is a ubiquitous hormone capable of exerting cardio- and vasculo-protective effects. Here we identify a novel protective action of adenosine A₃ receptors in skeletal muscle. Adenosine A₃ receptor-selective agonists reduced skeletal muscle injury in a mouse hindlimb ischemia/reperfusion model, an effect that was blocked by the A₃ receptor antagonist MRS1191 but not by A₁ receptor antagonist DPCPX. The A₁ receptor agonist was also able to exert a cytoprotective effect that was selectively blocked by DPCPX. The protection induced by A₃ receptor agonist was completely abrogated in phospholipase C $\beta 2/\beta 3$ -null mice while that caused by A₁ agonist remained unaffected in these animals. The protection by A₁ and A₃ receptor agonists was extended to the β -sarcoglycan deficiency-induced skeletal myopathy animals in which exercise-induced skeletal muscle injury was significantly attenuated by adenosine A₃ but not A₁ receptor agonist. Thus, adenosine A₃ receptors are capable of mediating a generalized skeletal muscle protective effect. The data add to the cyto-protective actions of adenosine and suggest that these receptors are potentially novel therapeutic targets in ameliorating skeletal muscle injury.

The complete detailed description of the study, methods, results, discussion, figures and references are shown in the attached Appendix I. Overall, the study completed two of the original tasks of the Statement of Work in that it established a quantitative model of ischemia-reperfusion injury using a mouse hindlimb model and it defined adenosine A₃ receptors as one of the skeletal muscle protective adenosine receptors. The study also confirms previous observation that the A₁ receptor is also cyto-protective at the skeletal muscle. Thus, agonists acting at this receptor represent a novel therapeutic target for treating skeletal muscle ischemia-reperfusion injury.

The clinical study received regulatory approval during this last year. Preparative works were performed to establish the clinical protocols and to train personnel. Three projects were initiated and their current status is outlined below.

The first project titled "Circulating adenosine levels before and after IV persantine" received regulatory approval from the US Army Medical Research and Materiel Command Human Subjects Research Review Board on 9/9/2005. Twenty two subjects were consented but only 20 were actually enrolled because we could not draw blood from two subjects. Please see enclosed

Appendix II for specific clinical details. Work is currently ongoing to quantify the ability of dipyridamole to inhibit the radio-labeled uridine uptake via the adenosine transporter into the red blood cells and to inhibit agonist-induced platelet aggregation. Polymorphisms of the transporter gene will be determined once we collected 48 subjects (48 will allow a complete sequencing run) and their relationships to the dipyridamole-mediated inhibition studied.

The second project is titled “Persantine: Variation in Response” and received regulatory approval in May, 2006. Preparatory work has been performed and personnel trained to carry out this study. To date, five subjects have been contacted. But for various reasons outlined in Appendix III, no subject has been enrolled.

The third project titled “Equilibrative Nucleoside Transporters and Hypertension: A Pilot Project” received regulatory approval in August of 2006. Two subjects were enrolled. Work is now ongoing to continue recruiting subjects. Once 48 subjects are recruited, we will sequence the transporter gene for polymorphisms.

KEY RESEARCH ACCOMPLISHMENTS:

- 1. Establishment of a quantitative hindlimb ischemia-reperfusion injury model in the mouse;**
- 2. Confirming that the adenosine A₁ receptor is cyto-protective against ischemia-reperfusion injury in skeletal muscle;**
- 3. Discover for the first time that the adenosine A₃ receptor is cyto-protective against ischemia-reperfusion injury in skeletal muscle;**
- 4. Begin to delineate the signaling mechanism mediating the protective effect of A₁ and A₃ receptors by showing that phospholipase C beta mediates the cytoprotection by A₃ but not A₁ receptor agonists;**
- 5. Establish clinical research protocols and obtain regulatory IRB approvals for three clinical projects**

REPORTABLE OUTCOMES:

An abstract has been submitted to Experimental Biology 2007 as follows.

A Novel Skeletal Muscle Cytoprotective Action of Adenosine A₃ Receptors

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A manuscript with the same title and authors are being prepared for submission.

CONCLUSION:

The importance of the work is that it will allow the development of novel method to treat skeletal muscle ischemia-reperfusion injury and has already identified a potentially new therapeutic target for treating of skeletal muscle injury. A dual benefit is that this target, the adenosine A₃ receptor, is also capable of exerting a protective effect in the heart. It is envisioned that agonists

at this receptor may exert a beneficial effect in both heart and skeletal muscle protection. Method or composition to administer adenosine A₃ agonists may become a novel way to treat skeletal muscle injury.

REFERENCES

All references for the manuscript are listed in Appendix I.

APPENDICES

Please see Appendices I through III

SUPPORTING DATA

For figures and legends, please see Appendix I.

A Novel Skeletal Muscle Cytoprotective Action of Adenosine A₃ Receptors

By

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Adenosine is a ubiquitous hormone capable of exerting cardio- and vasculo-protective effects. Here we identify a novel protective action of adenosine A₃ receptors in skeletal muscle. Adenosine A₃ receptor-selective agonists reduced skeletal muscle injury in a mouse hindlimb ischemia/reperfusion model, an effect that was blocked by the A₃ receptor antagonist MRS1191 but not by A₁ receptor antagonist DPCPX. The A₁ receptor agonist was also able to exert a cytoprotective effect that was selectively blocked by DPCPX. The protection induced by A₃ receptor agonist was completely abrogated in phospholipase C β 2/ β 3-null mice while that caused by A₁ agonist remained unaffected in these animals. The protection by A₁ and A₃ receptor agonists was extended to the β -sarcoglycan deficiency-induced skeletal myopathy animals in which exercise-induced skeletal muscle injury was significantly attenuated by adenosine A₃ but not A₁ receptor agonist. Thus, adenosine A₃ receptors are capable of mediating a generalized skeletal muscle protective effect. The data add to the cyto-protective actions of adenosine and suggest that these receptors are potentially novel therapeutic targets in ameliorating skeletal muscle injury.

Ischemia and reperfusion can cause significant skeletal muscle injury. Skeletal muscle is the most vulnerable tissue in the extremities (1,2). Trauma, autogenous skeletal muscle transplantation, surgical incision, vascular clamp application during vascular surgery or musculoskeletal reconstructive surgery as well as sustained strenuous exertion can also induce skeletal muscle damage with deleterious systemic consequences (3-14).

Reperfusion of the ischemic muscles can cause not only local but also distant organ injury with serious systemic consequences. Such injury can result from cardiopulmonary bypass, vascular cross clamping, or transplantation (13-15). Neutrophil infiltration and activation in the lungs can result from ischemia and reperfusion of the hindlimb (16, 17). Thus, protection of skeletal muscle from ischemia and reperfusion injury is an important therapeutic goal in ameliorating both local and remote muscle and organ injury.

Although various measures such as tissue-preserving solution and cold immersion are used to preserve liver, pancreas, and the kidney (18, 19), an effective method or agent to protect skeletal muscle from ischemia/reperfusion injury is lacking. Ischemic preconditioning can provide potent protection of the heart muscle (20, 21). Recent

evidence also suggests a cytoprotective effect of ischemic preconditioning in protecting skeletal muscle from ischemia/reperfusion injury (22-25). Similar to cytoprotection of the heart, adenosine is implicated in mediating the protective effect of preconditioning in skeletal muscle (24-25). Direct infusion of adenosine can mimic the effect of preconditioning in reducing skeletal muscle injury (24). Adenosine is an important regulatory agent that exerts its cytoprotective effect via activation of its receptors. Activation of either adenosine A₁ (26-28) or A₃ (29-31) receptors can mediate potent anti-ischemic cardioprotective effect. However, identity of the adenosine receptor subtype mediating skeletal muscle protection and its underlying signaling mechanism are not well understood.

Recent studies showed that R-PIA, a putative adenosine A₁ receptor-selective agonist, exerted an anti-ischemic effect in a pig latissimus dorsi muscle flap model (25). An adenosine A₁ receptor-selective antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), blocked the protection by adenosine in this model. These data implicated a role for adenosine A₁ receptors in mediating the protection against ischemia/reperfusion injury in skeletal muscle. However, the role of other adenosine receptor subtypes in protecting the skeletal muscle is not known. Activation of adenosine A₃ receptor has been shown to protect the myocardium against ischemia and reperfusion injury (29-31). However, mast cells express adenosine A₃ receptors (32, 33) whose activation can stimulate inflammation with potentially deleterious effect on skeletal muscle. A systematic investigation of the cytoprotective role of adenosine A₁ and A₃ receptors in skeletal muscle, with full pharmacological characterization using selective agonists and antagonists, is needed. A genetic non-pharmacological approach in determining the role

of adenosine receptor subtypes and its signaling mechanism at skeletal muscle protection is lacking.

To study these questions, we established a quantitative model in which skeletal muscle injury can be calculated using a mouse hindlimb ischemia/reperfusion preparation.

Ischemia followed by reperfusion resulted in significant limb skeletal muscle injury, as quantified by an increase in the Evans Blue Dye (EBD) staining of the skeletal myocytes (Fig 1A). Administration of a nonselective adenosine receptor agonist N⁶-1-(phenyl-2R-isopropyl)-adenosine (R-PIA) prior to ischemia and reperfusion caused a significant reduction in the extent of injury (Fig. 1B). In identifying the adenosine receptor that mediates this protective effect, a highly A₁ receptor-selective agonist, 2-chloro-N⁶-cyclopentyladenosine (CCPA), was found to induce a large decrease in the extent of muscle injury (Fig. 1C). The A₁ receptor-selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) completely abrogated the cytoprotective response to CCPA (Fig. 1D and E) while the adenosine A₃ receptor-selective antagonist MRS1191 did not affect the CCPA-mediated protection (Fig. 1F). These studies provided convincing pharmacological evidence that the adenosine A₁ receptor is capable of protecting skeletal muscle from ischemia/reperfusion injury and are consistent with conclusion by others (25). Another adenosine receptor known for its myocardial cytoprotective ability, the A₃ receptor, was also able to mediate a potent skeletal muscle protective effect. The A₃ receptor agonist 2-chloro-N⁶-(3-iodobenzyl)adenosine-5'-N-methyluronamide (CI-IBMECA) can induce a significant reduction in the number of EBD-positive cells (Fig. 2A). This reduction was sensitive to antagonism by MRS1191 (Fig. 2B and D) but not by DPCPX (Fig. 2C and E). These data linked each agonist's protective response to its

specific receptor and demonstrated for the first time that the adenosine A₃ receptor is capable of mediating a potent skeletal muscle protective effect.

The mechanism by which adenosine receptors mediate this protective response was further illustrated by the testing the agonist's effect in phospholipase C $\beta 2/\beta 3$ (PLC $\beta 2/\beta 3$)-null mice. CI-IBMECA-induced protection was completely abrogated in PLC $\beta 2/\beta 3$ -null mice while that induced by CCPA remained unaffected (Fig. 3). To rule out a nonspecific effect associated with CI-IBMECA as the reason for its lack of cytoprotective effect in PLC $\beta 2/\beta 3$ knockout animals, the protection mediated by a structurally different and adenosine A₃ receptor-selective agonist, MRS3558 (45), was investigated. MRS3558 also caused a large reduction in the EBD-positive skeletal muscle cells. This protection was completely lost in the PLC $\beta 2/\beta 3$ -null mice (Fig. 3). These data showed that the A₃ receptor, but not the A₁ receptor, signals via the beta isozyme of PLC to exert its skeletal muscle protective effect and provided further evidence for a distinct cytoprotective property of each adenosine receptor.

Recent studies showed that adenosine A_{2A} receptor serves an important non-redundant role in suppressing immune and lymphoid cells and thus in protecting against inflammatory tissue damage (34-36). Activation of adenosine A_{2A} receptors on the CD4⁺ T cells mediated potent protection against renal ischemia-reperfusion injury (37). The cytoprotective action of adenosine A₁ or A₃ receptors in skeletal muscle is independent of the salutary effect of the A_{2A} receptor on immune cells since the effects of A₁ or A₃ receptor-selective agonists were specifically blocked by their respective antagonists. The adenosine A₁ receptor appears limited in density, distribution, or function in the vascular or immune cells. Activation of the A₃ receptor in rodent immune cells such as the mast

cells is pro-inflammatory, in studies of myocardial injury and protection, actually damaging (38, 39). Genetic absence or antagonism of adenosine A₃ receptors augmented increase in coronary flow or hypotension mediated by adenosine or A_{2A} receptor agonist (40, 41), pointing to a vaso-constrictive role of the vascular A₃ receptor. Activated mast cells and neutrophils mediate skeletal muscle ischemia/reperfusion injury (42-44). Therefore, activation of the vascular or immune cell adenosine A₃ receptor is unlikely beneficial in the present in vivo skeletal muscle ischemia/reperfusion model. The beneficial in vivo effect of A₃ receptor activation in skeletal muscle protection is the balance of a net salutary action at non-vascular or immune tissues, likely the skeletal muscle itself, over the detrimental A₃ effect exerted in the vascular or immune cells. Overall, the present data demonstrated for the first time that activation of the adenosine A₃ receptor, likely those of the skeletal muscle, can exert a potent protection of the skeletal muscle against injury caused by ischemia/reperfusion or by excessive exercise.

Figure Legends

Figure 1. Cytoprotective action of adenosine in a quantitative model of mouse hindlimb ischemia and reperfusion injury. Adult wild type mice were injected with (A) sterile vehicle (0.1 % DMSO in phosphate-buffered saline, pH 7.4) or various adenosine receptor agonists (B, C) or antagonist (D, F). (A) Following ischemia and reperfusion, skeletal muscle showed a significant uptake of Evans Blue Dye (EBD) in vehicle-treated mice (first part of Fig. 1A, representative of 7 mice). The contra-lateral leg not subjected to ischemia-reperfusion showed virtually no EBD uptake. In the second part of figure 1A, the same section was stained with rabbit polyclonal anti-skeletal muscle actin antibodies followed by staining with goat anti-rabbit IgG conjugated with FITC. (B): The nonselective adenosine agonist R-PIA caused a large reduction in the EBD-stained area (see both first and second parts to this figure, representative of 6 mice). (C): A highly A₁ receptor-selective agonist CCPA decreased the %EBD-positive area (representative of 12 mice); (D): this reduction was reversed in mice injected with an A₁ receptor-selective antagonist DPCPX before CCPA (9 mice). (E): Average EBD staining (\pm SD) of skeletal muscle sections in vehicle- and adenosine analogs-treated were quantified by blinded observers (n=8 mice for DPCPX alone). (F): Conversely, MRS1191 could not block the CCPA-induced protection (n= 23 mice for MRS1191 plus CCPA; n= 15 mice for MRS1191 alone). *P<0.05.

Figure 2. Adenosine A₃ receptors are capable of mediating a potent anti-ischemic protective response distinct from that mediated by the A₁ receptor. (A): Adult WT mice

injected with an A₃ receptor-selective agonist Cl-IBMECA exhibited a significant reduction in the % EBD-stained muscle (8 mice). The first part corresponded to EBD-stained muscle while the second part was FITC-stained muscle. (B) and (D): This protection was reversed by the sequential injection of an A₃ receptor-selective antagonist MRS1191 and of Cl-IBMECA (22 mice). (C) and (E): However, the injection of A₁ receptor-selective antagonist DPCPX did not prevent the Cl-IBMECA-evoked protection (9 mice). Thus, the protective effect of CCPA and Cl-IBMECA was mediated specifically by its respective adenosine receptors. (D and E): Average EBD staining (\pm SD) of skeletal muscle sections in vehicle- and adenosine analogs-treated were quantified by blinded observers. *P<0.05.

Figure 3. Adenosine A₃ receptors signal through PLC β 2/ β 3 to cause its anti-ischemic skeletal muscle protective response. Adult PLC β 2/ β 3 null-mice were injected with vehicle (n=9 mice), Cl-IBMECA (8 mice), MRS3558 (9 mice) or CCPA (10 mice), subjected to ischemia and reperfusion, and the extent of skeletal muscle injury was subsequently quantified as in WT mice. PLC β 2/ β 3 null-mice not subjected to ischemia/reperfusion or skeletal muscle obtained from the contra-lateral limb not subjected to ischemia/reperfusion did not show any EBD staining. (A): Vehicle-injected PLC β 2/ β 3 null-mice showed similar extent of EBD staining as did vehicle-injected WT mice (vehicle-injected WT not shown). (B): CL-IBMECA did not reduce the % EBD-stained skeletal muscle in PLC β 2/ β 3 null-mice while (C) CCPA was effective in preventing the ischemia/reperfusion-induced injury in these mice. (D): A structurally unrelated A₃ receptor agonist MRS3558 was also able to reduce skeletal muscle injury in WT mice (representative of 8 mice). (E) This reduction persisted in the presence of the

A₁ receptor antagonist DPCPX in WT animals (n=10 mice), in support of the concept that the protective response to this novel agonist was mediated by the A₃ but not the A₁ receptor. (F) The protective effect of MRS3558 was completely abrogated in PLC β2/ β3 null-mice, providing further evidence for the hypothesis that the A₃ receptor signals via PLCβ to achieve its cytoprotective effect. (G) Average EBD staining (\pm SD) of skeletal muscle sections in vehicle- and adenosine analogs-treated PLC β2/ β3 null-mice were quantified by blinded observers. *P<0.05.

Materials and Methods

Mouse Hindlimb Ischemia and Reperfusion Model

Upon completion of anesthetic sedation (pentobarbital 50 mg/kg was given i.p), the right or left hindlimbs (used randomly) of 2 ½ to 3-month WT (C57BL6) or PLC $\beta 2/\beta 3$ -knockout mice weighing about 25 gm were elevated briefly and then subjected to ischemia induced by placement of a constrictor band (Latex O-Rings, Miltex Instruments, York, Pa) above the greater trochanter using a McGiveney Hemorrhoidal Ligator (7 inches long, Miltex). After 90 minutes of warm ischemia (temperature at 37° C), the constrictor was removed to allow reperfusion which was for 24 hrs. They were continuingly placed on a 37° C warming pad (Physitemp Instruments Inc, Clifton, NJ) during the reperfusion. After the mice were killed by anesthetic overdose, the gastrocnemius muscle was quickly frozen, cut into three pieces, and embedded in Shandon Cryomatrix (polyvinyl alcohol 10%, polyethylene Glycol 4%, Anatomical Pathology U.S.A., Pittsburgh, PA). Each piece was then frozen sectioned into 10 μ m slices on a Thermo Electron/Shandon Cryotome (Anatomical Pathology), fixed in ice cold acetone, air dried, washed in PBS.

Quantification of Injured Skeletal Muscle

Each 10 μ m section was then stained with rabbit polyclonal anti-skeletal muscle actin antibodies (ab15265, Abcam Inc, Cambridge, Ma) and goat polyclonal anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC). Slices were mounted, viewed on

fluorescent microscopy (EBD-positive cells via a DM580 band pass filter 510-560 nm with emission of 590 nm; FITC cells via a DM510 filter of 450-490 nm with emission at 520 nm). The same slices were viewed and their images captured via the two filters for quantification of muscle injury as follows. Images were acquired and stored as a JPEG file with a Macrofire camera (Macrofire 1.0, Optronics, Goleta, CA). EBD-positive cells were quantified (ImageProPlus, version 5.0, Media Cybernetics, Inc., Silver Spring, MD; ref.). The percentage of EBD-positive areas was calculated by dividing the area of staining by the total muscle cells which was defined as areas stained by anti-skeletal muscle actin.

Protocol of Adenosine Receptor Agonist and Antagonist Administration

Adenosine receptor agonists (30 μ M except 15 μ M for R-PIA), antagonist (DPCPX at 0.2 mg/kg, MRS1191 at 25 μ M) or vehicle (0.1 %DMSO in phosphate-buffered saline) was administered in a sterile 0.1 ml volume by intra-peritoneal (i.p.) injection 2 hr before induction of ischemia. Evans Blue dye (EBD, 1% wt/vol solution to yield 1 mg of EBD/10 gm body weight) was also given via a separate i.p. injection 2 ½ hr before induction of ischemia. When both antagonist and agonist were administered, the antagonist was given 30 min before the agonist. Data were shown as mean \pm standard deviations. One-way ANOVA followed by posttest comparison was used to analyze the statistical significance of differences in more than two groups.

Materials and Chemicals

The adenosine analogs 2-chloro-N⁶-cyclopentyladenosine (CCPA), 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), 2-chloro-N⁶-(3-iodobenzyl)adenosine-5'-N-methyluronamide (CI-IB-MECA), N⁶-R-phenyl-2-propyladenosine (R-PIA), and 3-ethyl 5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS 1191) were from Sigma Chemicals (St. Luis, MO). MRS3558 ((1'R,2'R,3'S,4'R,5'S)-4-{2-chloro-6-[(3-chlorophenylmethyl)amino]purin-9-yl}-1-(methylaminocarbonyl)bicyclo[3.1.0]hexane-2,3-diol) was synthesized as previously described (45).

PLC β 2/ β 3- and β -sarcoglycan-deficient mice

PLC β 2/ β 3-null mice were bred as previously described (). β -sarcoglycan-deficient mice were developed and bred according to previously described method (). C57BL6 mice were obtained from Jackson Laboratories (Bar Harbor, Maine). All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for research and approved by the IACUC of University of Connecticut Health Center.

References

1. Ecker P and Schnackerz K. Ischemic tolerance of human skeletal muscle. *Ann Plast Surg.* 1991;26:77-84.
2. Blaisdell FW. The pathophysiology of skeletal muscle ischemia and perfusion syndrome: a review. *Cardiovasc Surg.* 2002;10:620-630.
3. Carrol CMA, Carrole SM, Overgoor MLE, Tobin G, Barker JH. Acute ischemic preconditioning of skeletal muscle prior to flap elevation augments muscle-flap survival. *Plastic reconstr Surg.* 1997;100:58-65
4. Haimovici H. Muscular, renal and metabolic complication of acute arterial occlusions: myonephpathic-metabolic syndrome. *Surgery* 1979;85:461-8.
5. Haimovici H. Vascular sutures and anastomosis. In: *Vascular Surgery*, ed. Boston AE, Blackwell, chap. 15, pp221-287, 2004.
6. Walker PM. Symposium on acute arterial insufficiency. 1. Pathophysiology of acute arterial occlusion. *Can J. Sur.* 1986;29:340-2.
7. Grace PA. Ischemia-reperfusion injury. *Br. J. Surg* 1994;81:637-647.
8. Haimovici H. Arterial embolism with acute massive ischemic myopathy and myoglobinuria. *Surgery* 1960;47:739.
9. Beyersdorf F, Unger A, Wildhirt A, Kretzer U, Deutschlander N, Kruger S, Matheis G, Hanselmann A, Zimmer G, Satter P. Studies of reperfusion injury in skeletal muscle: preserved cellular viability after extended periods of warm ischemia. *J. Cardiovasc Surg* 1991;32:664.

10. Tountas CP, Bergman RA. Tourniquet ischemia: ultrastructural and histochemical observations of ischemic human muscle and of monkey muscle and nerve. *J. Hand Surg [AM]* 1977;2:31-7.
11. Larsson J and Hultman E. The effect of long-term arterial occlusion on energy metabolism of the human quadriceps muscle. *Scand J Clin Lab Invest.* 1979;39:252-264.
12. Eckert P and Schnackerz K. Ischemic tolerance of human skeletal muscle *Ann Plast Surg* 1991;26:77-84.
13. Cotran RS, Kumar V, Collins T. eds. *Robbins Pathologic Basis of Disease*, 1999, pp1-30, Saunders, Philadelphia.
14. Gallin JJ, Snyderman R. in *Inflammation: Basic Principles and Clinical Correlations*, eds. Gallin JJ, Snyderman R, Fearon DT, Haynes BF, Nathan C, 1999, Lippincott Williams & Wilkins, Philadelphia. 1999, pp.1047-1061.
15. Gelman S. *Anesthesiology* 1995;82:1026-1060.
16. Lefer AM *Agents Actions* 1993;41 (Suppl):127-135.
17. Qiu F-H, Wada K, Stahl GL, Serhan CN. IMP and AMP deaminase in reperfusion injury down-regulates neutrophil recruitment. *Proc. Natl. Acad. Sci. USA* 2000; 97:4267-4272.b
18. Southard JH, Belzer FO *Organ preservation. Annu. Rev. Med.* 1995;46:235.
19. Tsuchida T, Kato T, Yamaga M, Ikebe K, Oniki Y, Irie H, Takagi, K. The effect of perfusion with UW solution on the skeletal muscle and vascular endothelial exocrine function in rat hindlimbs. *J Surg. Res.* 2003;110:266-271.

20. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circ* 1986;74:1124-36.
21. Yellon DM, Downey JM. Preconditioning the myocardium: from cellular physiology to clinical cardiology. *Physiol. Rev.* 2003;*3:1113-1151.
22. Bushell AJ, Klenerman L, Taylor S, Davies H, Grierson I, Helliwell TR, Jackson MJ. Ischemic preconditioning of skeletal muscle. 1. Protection against the structural changes induced by ischemic/reperfusion injury. *J. Bone Joint Surg.* 2002;84-B:1184-8.
23. Bushell AJ, Klenerman L, Davies H, Grierson I, McArdle A, Jackson MJ. Ischemic preconditioning of skeletal muscle. 2. Investigation of the potential mechanisms involved. *J. Bone Joint Surg.* 2002;84-B:1189-93.
24. Lee HT, Schroeder CA, Shah PM, Babu SC, Thompson CI, Belloni FL. Preconditioning with ischemia or adenosine protects skeletal muscle from ischemic tissue reperfusion injury. *J. Surg Res.* 1996;63:29-34.
25. Pang CY, Neligan P, Zhong A, He W, Xu H, Forrest CR. Effector mechanism of adenosine in acute ischemic preconditioning of skeletal muscle against infarction. *Am. J. Physiol (Regulatory Integrative Comp. Physiol. 42).* 1997; 273:R887-R895.
26. Fredholm BB, Ijzerman AP, Jacobson KA, Klotz KN, Linden J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.* 2001;53:527-552.

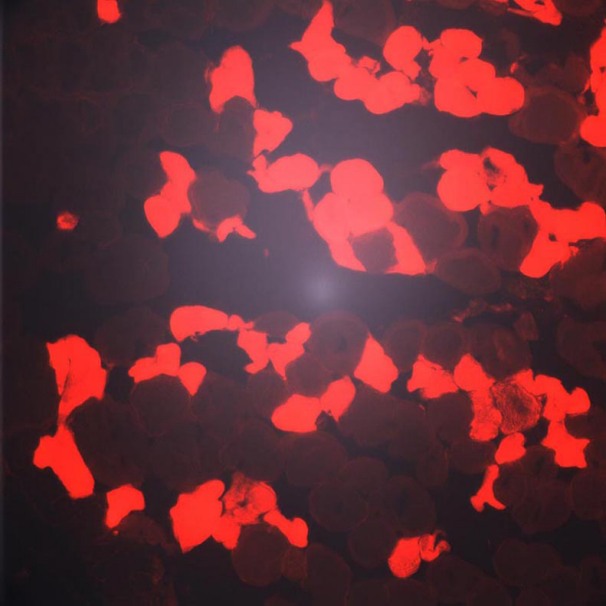
27. Headrick JP, Gauthier NS, Morrison R, Matherne GP. Cardioprotection by K₉ATP) channels in wild-type hearts and hearts overexpressing A₁-adenosine receptors. *Am. J. Physiol. Heart Circ Physiology* 2000;279:H1690-H1697.
28. Reichelt ME et al Genetic deletion of the A₁ adenosine receptor limits myocardial ischemic tolerance. *Circ Res.* 2005;96:363-367.
29. Liang BT, Jacobson KA. A physiological role of adenosine A₃ receptor: sustained cardioprotection. *Proc. Natl. Acad. Sci. U.S.A.* 1998;95: 6995-6999.
30. Auchampach JA, Ge Z-D, Wan TC, Moore J, Gross, GJ. A₃ adenosine receptor agonist IB-MECA reduces myocardial ischemia-reperfusion injury in dogs. *Am. J. Physiol. Heart Circ Physiol.* 2003;285:607-613.
31. Maddock HL, Mocanu MM, Yellon DM. Adenosine A₃ receptor activation protects the myocardium from reperfusion/reoxygenation injury *Am. J Physiol. Heart Circ Physiol.* 2002; 283:H1307-H1313.
32. Ramkumar V, Stiles GL, Beaven MA, Ali H. The A₃ adenosine receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells. *J. Biol. Chem.* 1993;268:16887-16890.
33. Tilley SL, Wagoner VA, Salvatore CA, Jacobson MA, Koller BH. Adenosine and inosine increase cutaneous vasopermeability by activating A₃ receptors on mast cells. *J. Clin Invest.* 2000;105:361-7.
34. Sitkovsky MV, Lukashev D, Apasov S, Kojima H, Koshiba M, Caldwell C, Ohta A, Thiel M. Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A_{2A} receptors. *Annu Rev Immunol* 2004;22:657-82.

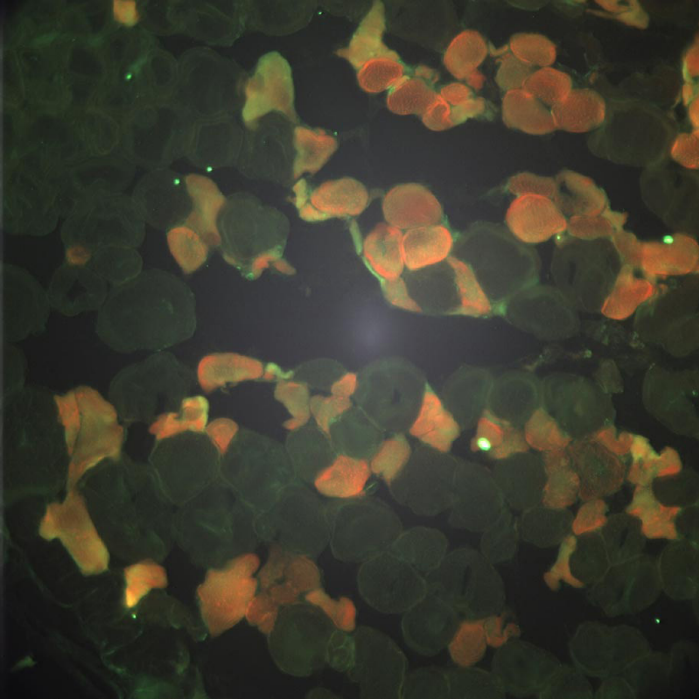
35. Lukashev D, Ohta A, Apasov S, Chen J-F, Sitkovsky M. Cutting edge: Physiologic attenuation of proinflammatory transcription by the Gs protein-coupled A_{2A} adenosine receptor in vivo. *The Journal of Immunology* 2004;173:21-24.
36. Sitkovsky M, Lukashev D. Regulation of immune cells by local-tissue oxygen tension: HIF1a and adenosine receptors. *Nature reviews/Immunology* 2005; 5:712-721.
37. Day Y-J, Huang L, Ye H, Li L, Linden J, Okusa MD. Renal ischemia-reperfusion injury and adenosine A_{2A} receptor-mediated tissue protection: The role of CD4⁺ T cells and IFN-g. *The Journal of Immunology* 2006;176:3108-3114.
38. Cerniway RJ, Yang Z, Jacobson MA, Linden J, Mathern GP. Targeted deletion of A₃ adenosine receptors improves tolerance to ischemia-reperfusion injury in mouse myocardium. *Am. J. Physiol. Heart Circ Physiol.* 2001; 281:H1751-H1758.
39. Harrison GJ. Effects of A₃ adenosine receptor activation and gene knockout in ischemic-reperfused mouse heart. *Cardiovasc Res* 2002;53:147-155.
40. Zhao Z, Makaritsis K, Francis CE, Gavras H, Ravid K. A role for the A₃ adenosine receptor in determining tissue levels of cAMP and blood pressure: studies in knock-out mice. *Biochim. Biophys. Acta.* 2000;1500:280-290/
41. Talukder MA, Targeted deletion of adenosine A₃ receptors augments adenosine-induced coronary flow in isolated mouse heart. *Am. J. Physiol. Heart Circ. Physiol.* 2002;282:H2183-H2189.

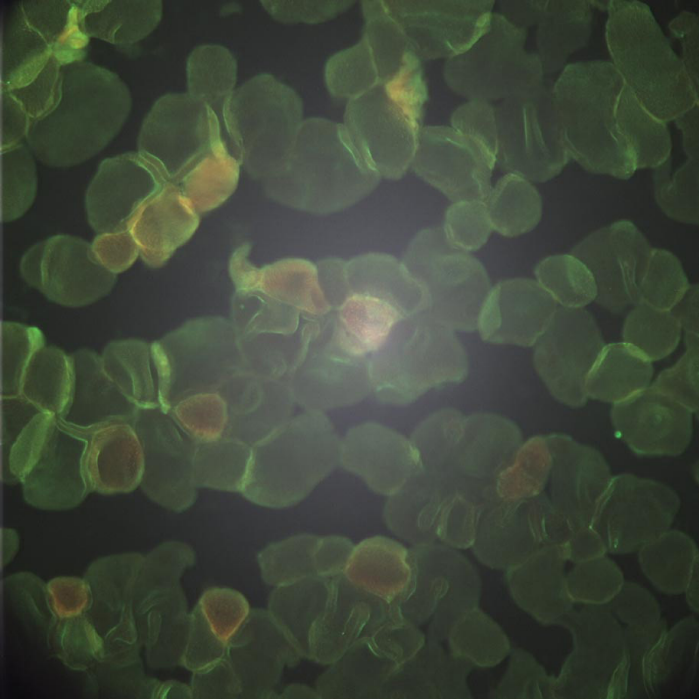
42. Bortolotto SK, Morrison WA, Han X-L, Messina A. Mast cells play a pivotal role in ischemia reperfusion injury to skeletal muscle. *Lab. Invest.* 2004; 1-9.
43. Formigli L, Lombardo LD, Adembri C, Brunelleschi S, Ferrari E, Novelli GP. Neutrophils and mediators of human skeletal muscle ischemia-reperfusion syndrome. *Hum Pathol* 1992; 23:627-634.
44. Fielding RA, Manfredi TJ, Ding W, Fiatarone MA, Evans WJ, Cannon JG. Acute phase response in exercise III. Neutrophil and IL-1b accumulation in skeletal muscle. *Am. J Physiol.* 1993;265:R166-R172.
45. Tchilibon, S., Joshi, B.V., Kim, S.K., Duong, H.T., Gao, Z.G., Jacobson, K.A. (N)-Methanocarpa 2,*N*⁶-disubstituted adenine nucleosides as highly potent and selective A₃ adenosine receptor agonists. *J. Med. Chem.*, 2005, 48:1745-1758

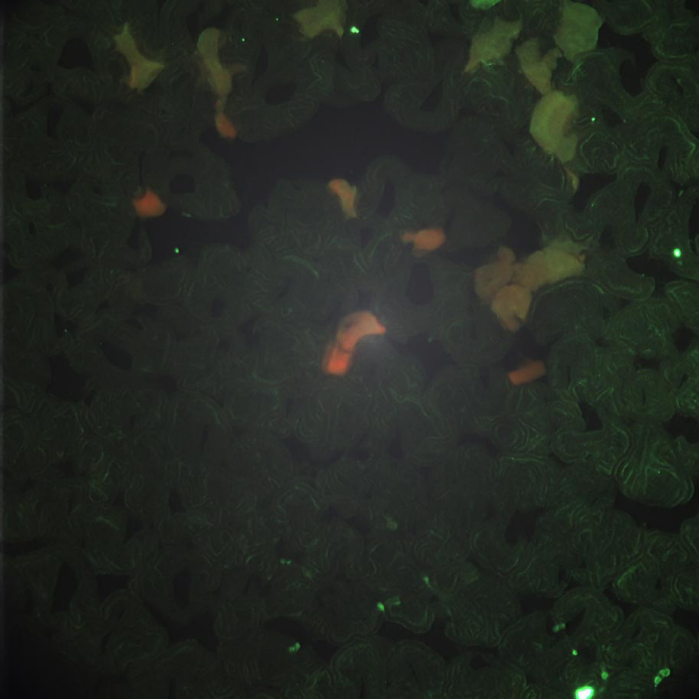
Pang CY, Forrest CR. Acute pharmacologic preconditioning as a new concept and alternative approach for prevention of skeletal muscle ischemic necrosis. *Biochem. Pharmacol.* 1995; 49:1023-1034.

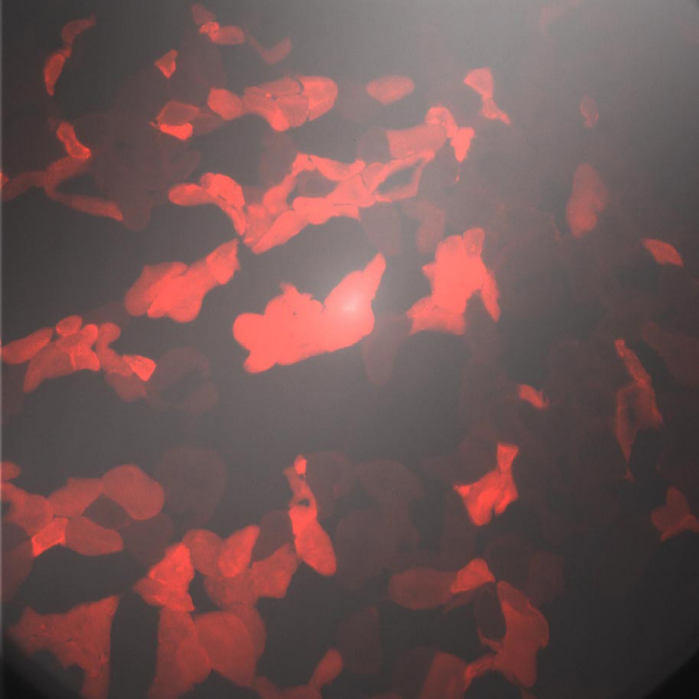
Austen WG, Zheng M, Chan R, Friend D, Hechtman HB, Carroll MC, Moore FD. Murine hindlimb reperfusion injury can be initiated by a self-reactive monoclonal IgM. *Surgery* 2004;136:401-6.

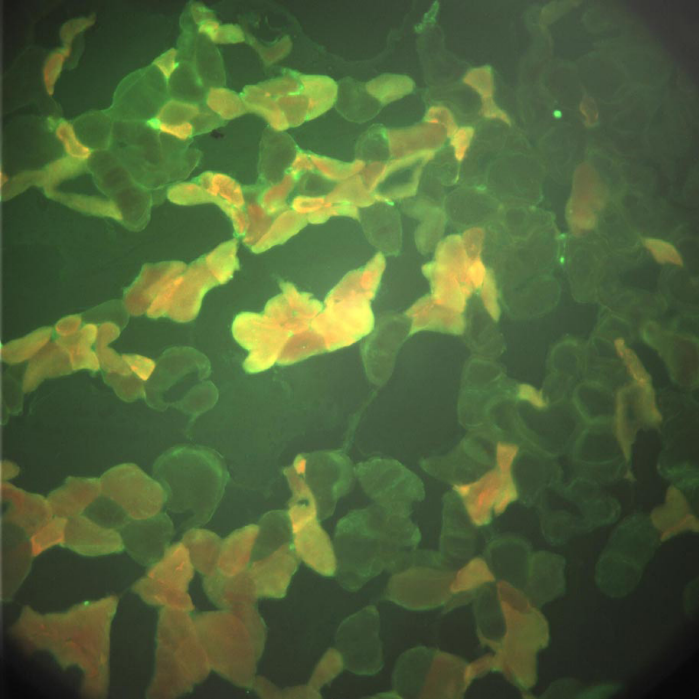






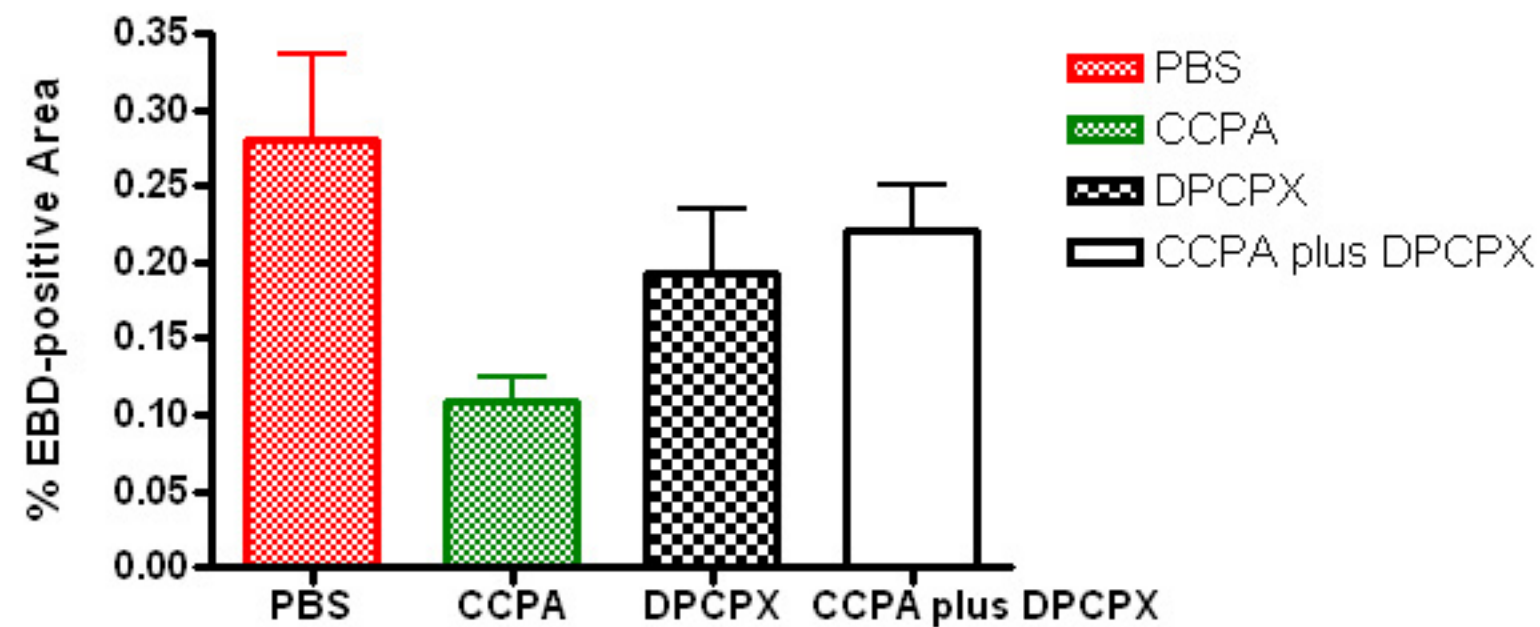






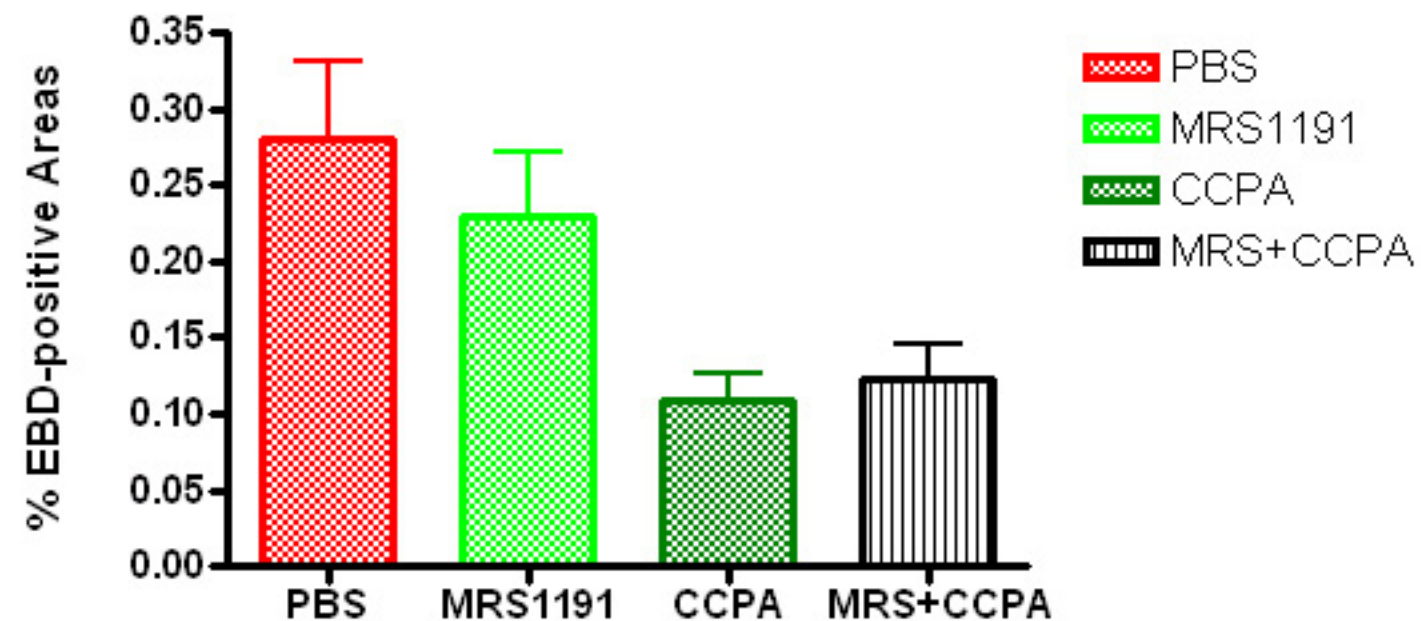
DPCPX Blocks CCPA-induced Protection from Ischemia/Reperfusion Injury in Skeletal Muscle

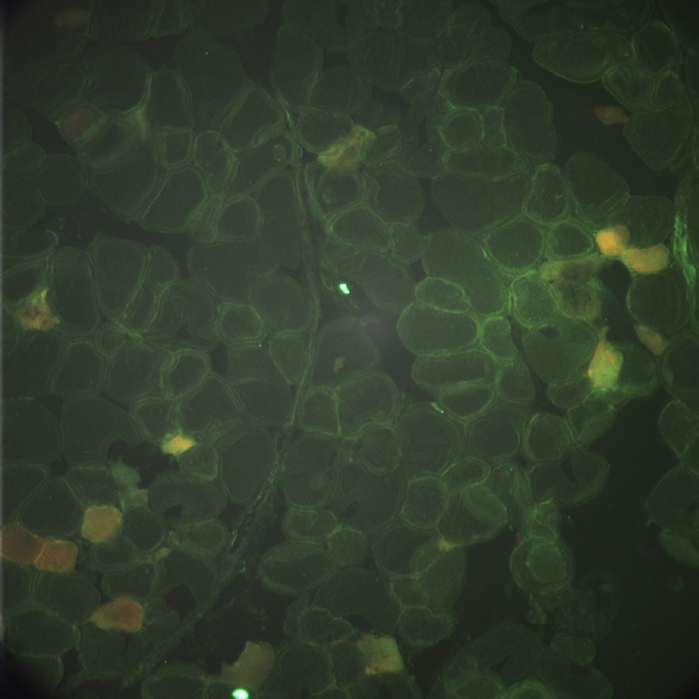
Fig 1E

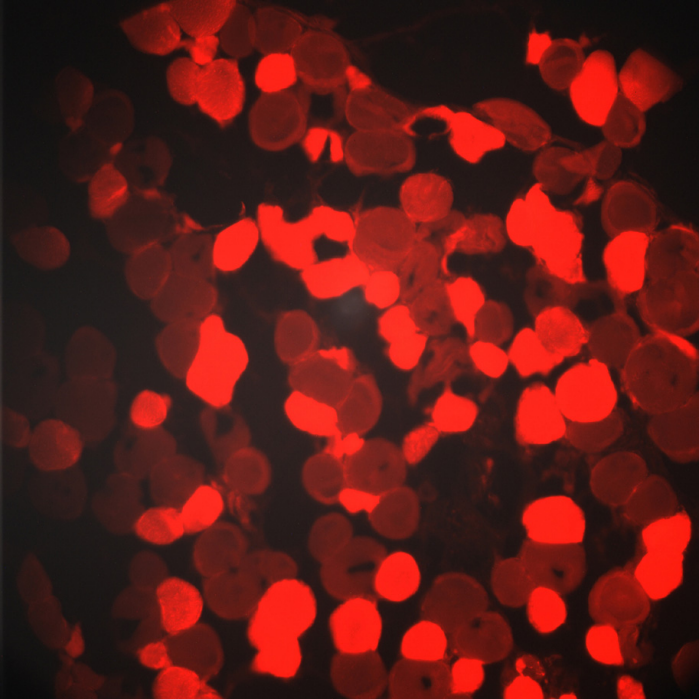


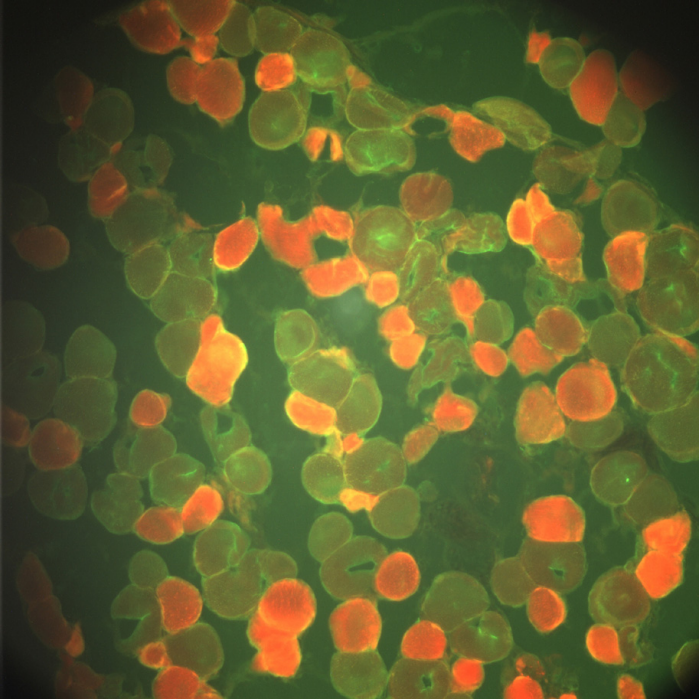
Adenosine A₃ Antagonist MRS1191 Did NOT Block the CCPA-induced Protection

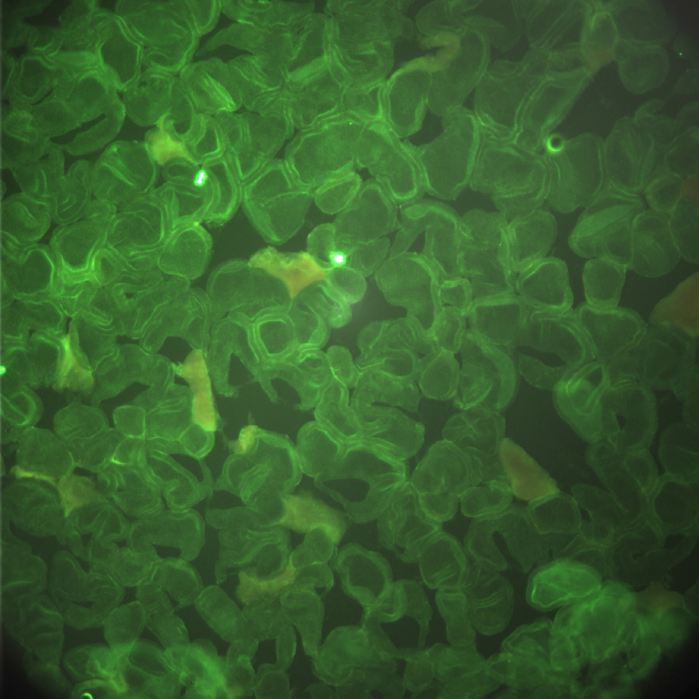
Fig. 1F





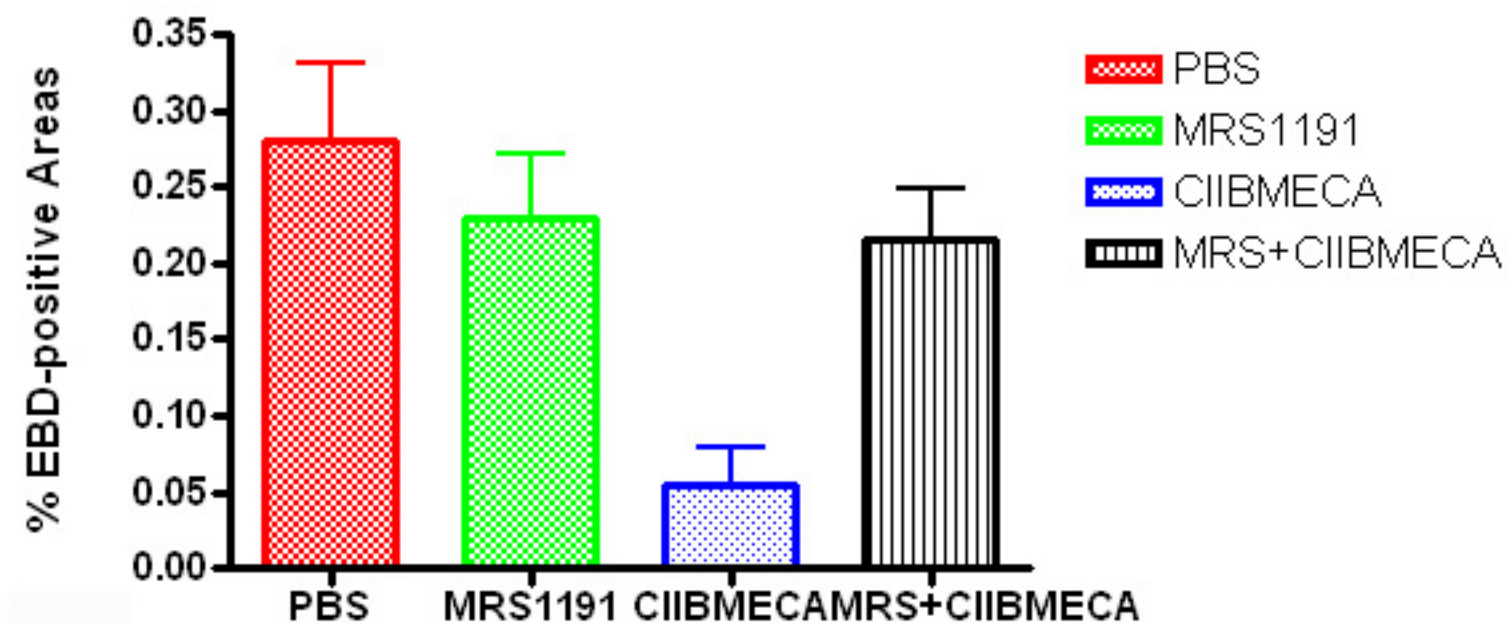




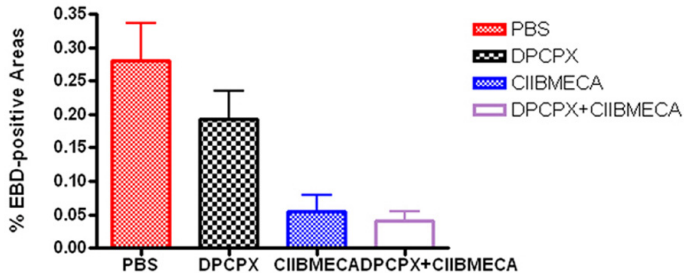


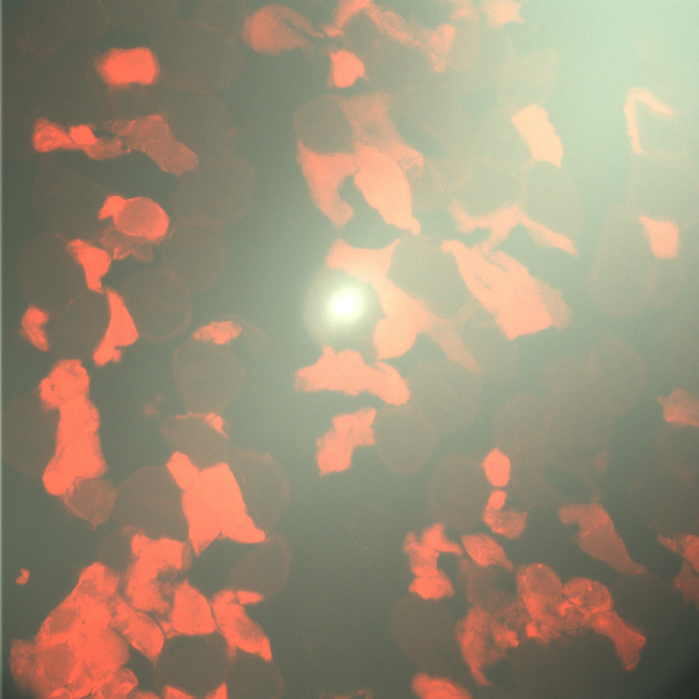
Adenosine A₃ Antagonist MRS1191 Blocked CI-IBMECA-induced Protection

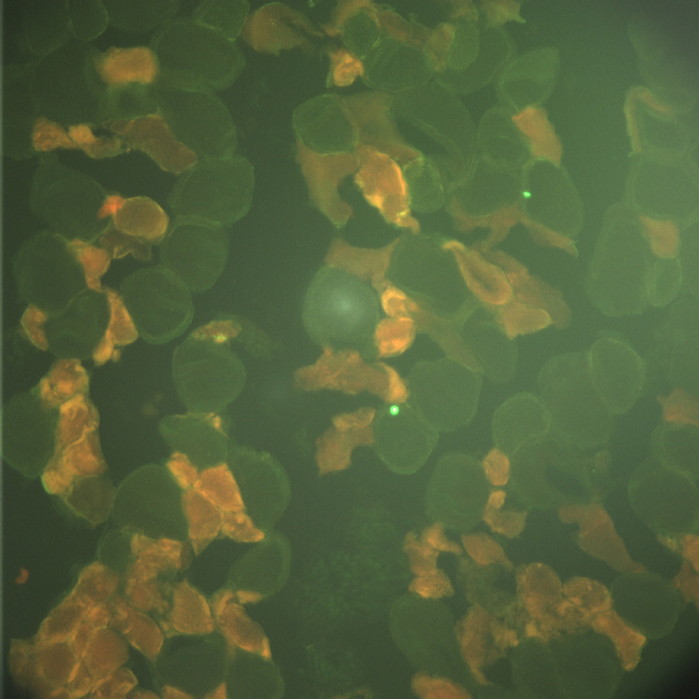
FIG. 2D

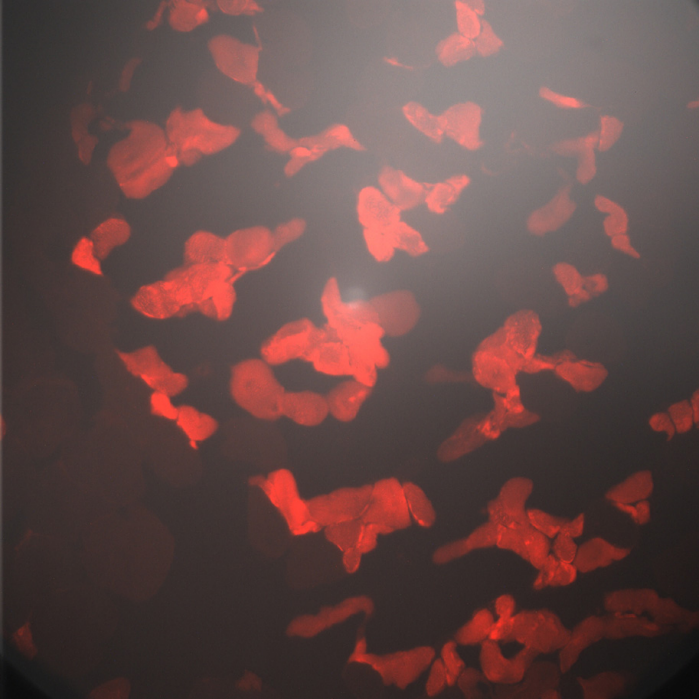


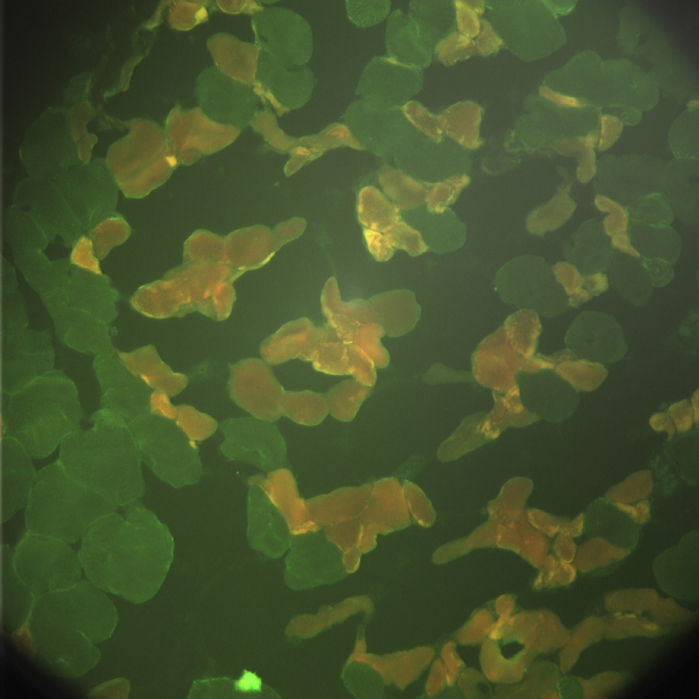
Adenosine A₃ Agonist Can Protect Skeletal Muscle From Ischemia/Reperfusion Injury **Fig. 2E**

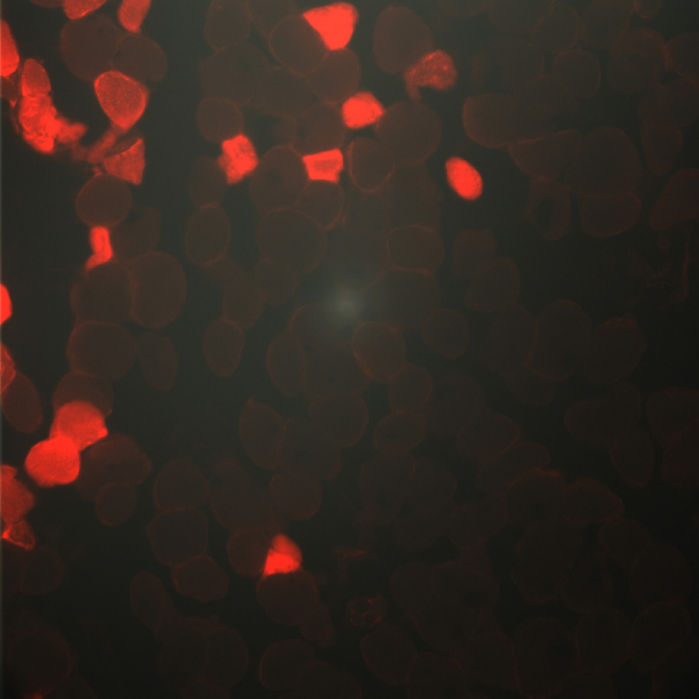


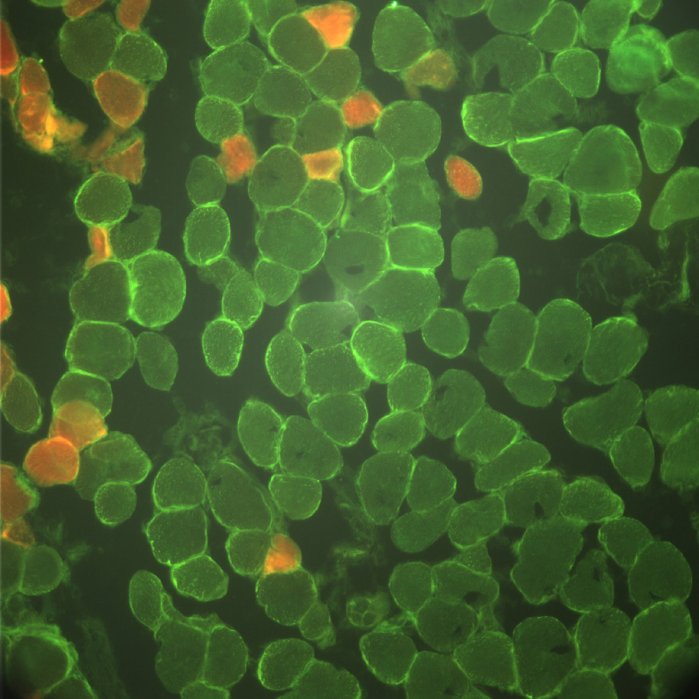


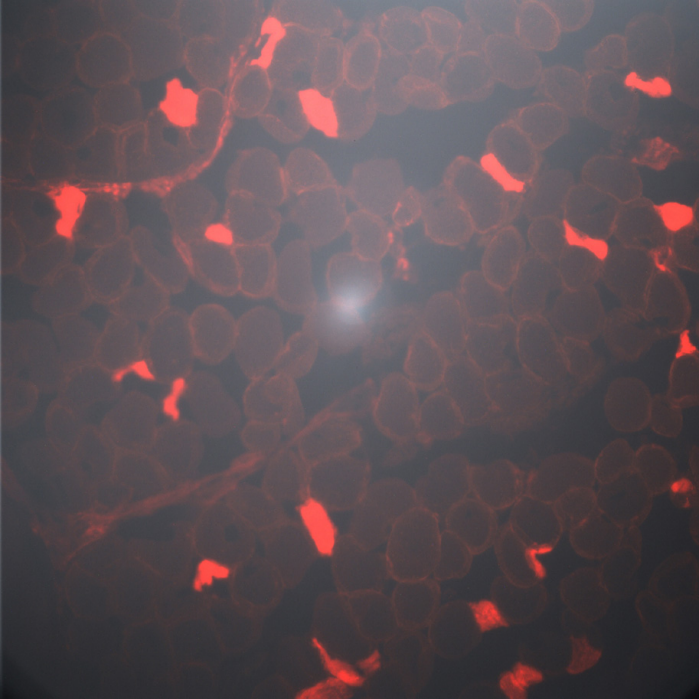


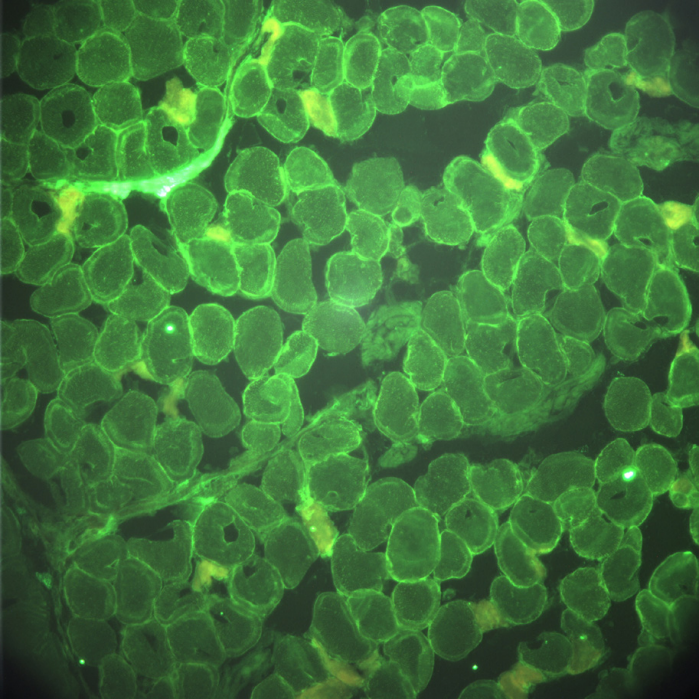


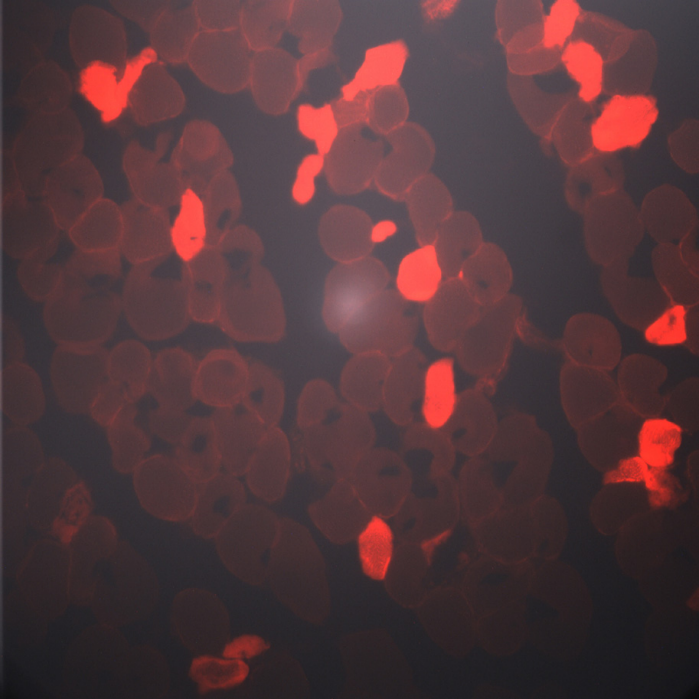


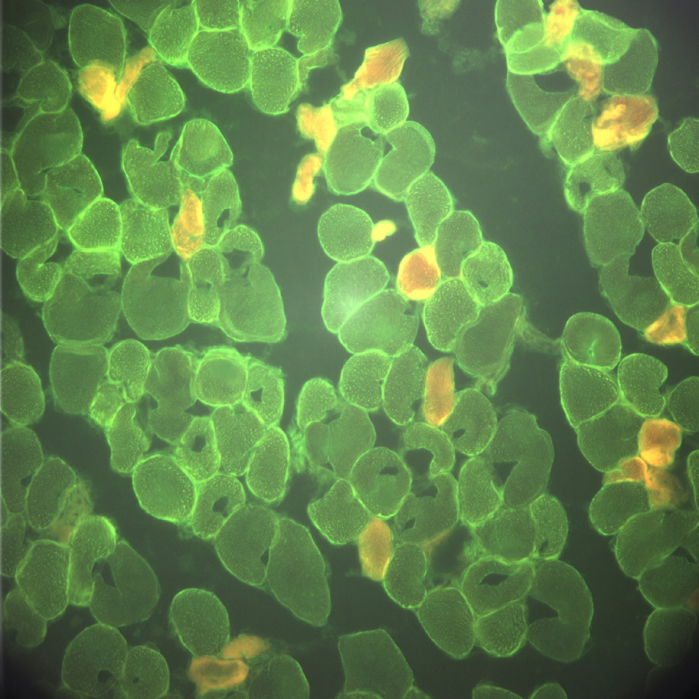


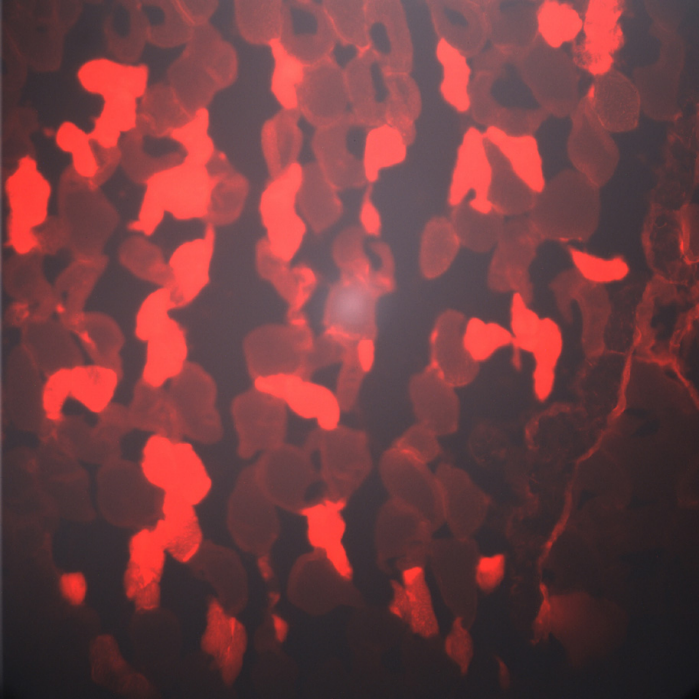


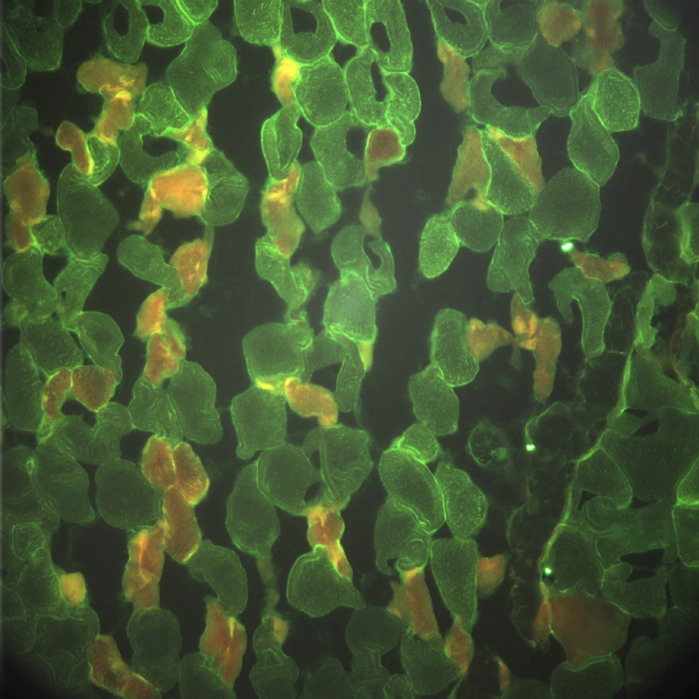




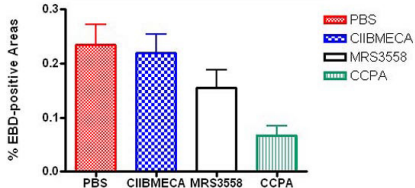








PLC $\beta 2/\beta 3$ deficiency abrogates A_3 but not A_1 receptor-mediated protection



Appendix II

Appendix II summarizes the clinical profile of subjects enrolled in the clinical protocol “Circulating adenosine levels before and after IV persantine”. The clinical profiles for these subjects are important because a specific clinical parameter, such as gender, race, hypertension or diabetes, may correspond to a specific genetic polymorphism of the adenosine transporter. In addition, the presence of a specific genetic polymorphism may correlate with an altered ability of dipyridamole to inhibit platelet aggregation or uridine uptake. Ability of dipyridamole to inhibit platelet function or uridine uptake represents an in vitro index of functional responsiveness. At this time, we continue to enroll subjects whose clinical profiles, functional responsiveness to dipyridamole, and genetic polymorphism will be obtained as the research progresses. Data and interpretation will be forthcoming.

Appendix III

Appendix III summarizes the status of the activity for the project “Persantine: Variation in Response”. It shows the reasons why five subjects consented could not be enrolled. Subjects will need to have both a normal coronary artery branch and a diseased branch. The normal branch allows assessment of coronary flow response to dipyridamole while the diseased branch allows determination of wall motion abnormality as a result of dipyridamole infusion. Some of the patients consented did not turn out to have this kind of anatomy while others declined to be enrolled.

Appendix II

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Appendix II

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Appendix II

[illegible]

Appendix II

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[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

**Persantine: Variation in Response
SCREEN FAILURE LOG**

Name/TO#:	Age:	DOB:	Ethnicity:	Screen Fx. Date
004 934701	80yr.	9-20-1925	white	Pt. declined 7-17-2006
005 079510	79yr.	9-20-1926	white	Pt. declined 8-21-2006
006 626022	63y	9-5-1943	latino	pt. did not speak English 8-21-2006
007 482652	34yr	5-7-1972	latino	pt. had clean arteries 8-23-2006
008 098475	74yr	6-7-1932	white	pt. had EJ fx.<30